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Prevention of Epstein-Barr Virus-Induced B-Cell Outgrowth by Interferon Alpha

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An in vitro system for determining the efficacy of interferon alpha (IFN-α) in preventing B-cell outgrowth due to Epstein-Barr virus (EBV) was developed. Unfractionated cord blood mononuclear cells, T-cell-depleted cord blood mononuclear cells, or adult T-cell-depleted mononuclear cells were exposed to IFN-α for 18 to 20 h followed by incubation with the B95-8 strain of EBV for 2 h. B-cell outgrowth was monitored by microscopic examination, [^{3}H]thymidine incorporation, and Epstein-Barr nuclear antigen detection. Cell density and viral inoculum both affected the sensitivity of outgrowth to IFN-α. IFN-α was most effective when added at each feeding after infection as well as before infection with EBV. The mean of the lowest IFN-α concentration tested at which transformation failed to occur after infection with the B95-8 strain of EBV at a 50% transforming dose of $10^{2.0}$ to $10^{3.0}$/ml was similar for unfractionated cord blood mononuclear cells, T-cell-depleted cord blood mononuclear cells, and adult T-cell-depleted mononuclear cells. The B95-8 strain and clinical EBV isolates required similar IFN-α concentrations to prevent outgrowth. In this system, IFN-α at pharmacologically achievable concentrations prevented EBV-induced B-cell outgrowth. These data indicate that IFN-α deserves further study as a potential therapeutic agent for EBV-induced syndromes.

Epstein-Barr virus (EBV), a lymphotropic human herpesvirus, is firmly established as the etiological agent of heterophile-positive infectious mononucleosis (6). EBV has also been implicated in the pathogenesis of African Burkitt's lymphoma (23), anaplastic nasopharyngeal carcinoma (12), and in some lymphomas arising in immunosuppressed patients (4, 18).

Since interferons possess both antiviral and antineoplastic properties, several investigators have examined the ability of interferons to inhibit EBV-induced B-cell transformation, often with conflicting results. Menezes et al. (11), using cord blood mononuclear cells (CBMCs), a large viral inoculum, and interferon alpha (IFN-α), found that IFN-α had no effect on Epstein-Barr nuclear antigen (EBNA) positivity or on outgrowth of transformed B-cells. In contrast, Lai et al. (8) found that filtered supernatants containing material with IFN-like properties (produced by challenge of EBV-sensitized leukocytes with EBV antigen) were able to prevent transformation of CBMCs by EBV. Thorley-Lawson (21) has reported that [^{3}H]thymidine uptake is inhibited in EBV-infected adult B-cells treated continuously with IFN-α, but that no inhibition occurs if cord blood B-cells are used. This was true even when 10,000 U of IFN-α per ml was used. Lvovsky et al. (10) investigated the sensitivity of EBV-induced B-cell transformation to IFN-α, IFN-β, and lymphoblastoid IFN. IFN-α and lymphoblastoid IFN inhibited transformation, whereas IFN-β did not. Most recently, Doetsch et al. (2) studied IFN-α, IFN-β, and core (2′-5′)-oligoadenylate and its cordycepin analog and reported that all four preparations were able to prevent cord and adult B-cell transformation by EBV.

In view of the need for more effective therapy for EBV-associated malignant and nonmalignant syndromes, as well as the conflicting statements concerning the in vitro utility of IFN-α in preventing EBV-induced outgrowth of B-cells, we studied its ability to prevent B-cell outgrowth in both cord blood and adult B-cells, using a standardized assay system. We also determined the sensitivity of B-cell outgrowth to IFN-α after infection with clinical EBV isolates.

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MATERIALS AND METHODS

Virus. The B95-8 marmoset lymphoblastoid cell line (kindly provided by David Thorley-Lawson) was used as the source of stock virus. It was maintained at 37°C in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 10% fetal calf serum (GIBCO), 250 μg of streptomycin per ml, 250 U of penicillin per ml, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (Research Organics, Inc., Cleveland, Ohio). Viral titers were determined by inoculating 10-fold dilutions of B95-8 supernatant fluid onto CBMCs and observing weekly for microscopic evidence of transformed B-cell outgrowth (22). Titers were calculated by the method of Reed and Muench (16), and aliquots were frozen at −70°C for later use.

Throat wash specimens were obtained by having renal allograft recipients and patients with heterophile-positive infectious mononucleosis gargle with RPMI 1640; saliva specimens were diluted 1:1 in RPMI 1640. Fetal calf serum, streptomycin, penicillin, and HEPES buffer were then added (in the concentrations previously cited) to throat wash and saliva samples, and the specimens were passed through a 0.45-μm filter (Millipore Corp., Bedford, Mass.) and frozen in aliquots at −70°C for subsequent use. Transforming titers were determined by the same technique used for B95-8 virus.

Cells. Adult peripheral blood or cord blood (obtained courtesy of the Beth Israel Hospital, Brookline, Mass.) was heparinized and diluted 1:1 in RPMI 1640 and then centrifuged over a Ficoll-Hypaque gradient. Mononuclear cells were harvested from the interface and washed twice in RPMI 1640. T-cell-depleted fractions were prepared by incubating the mononuclear cell fraction with neuraminidase-treated sheep erythrocytes and then centrifuging over a second Ficoll-Hypaque gradient. The T-cell-depleted fraction was harvested and washed twice in RPMI 1640.

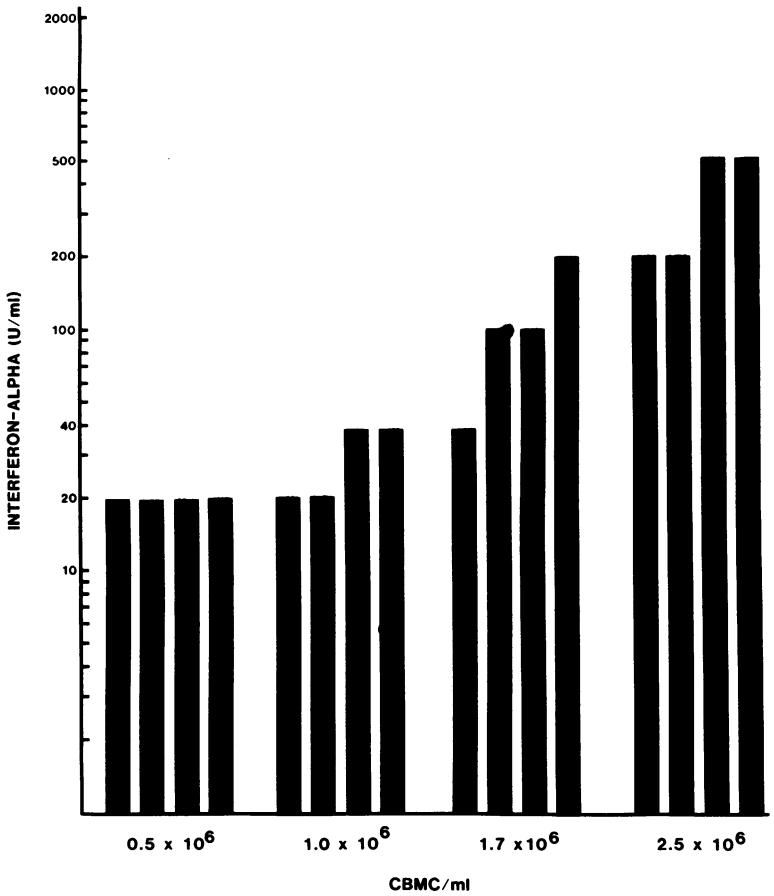

FIG. 1. Effect of CBMC concentration on IFN-α-mediated inhibition of EBV-induced transformed B-cell outgrowth. Cells used in this experiment were isolated from a single cord blood specimen. Each bar represents a single series of wells containing increasing concentrations of IFN-α. The bar terminates at the first IFN-α concentration at which outgrowth failed to occur. IFN-α concentrations of 0, 20, 40, 100, 200, 500, 1,000, and 2,000 U/ml were used in this and subsequent experiments.

IFN. Buffy coat-derived human IFN-α (1.1×10^6 U/mg of protein) was provided by K. Cantell and assayed by a cytopathic effect reduction method, using GM2504 cells (Human Genetic Mutant Cell Repository, Camden, N.J.) and vesicular stomatitis virus. National Institutes of Health preparation G023-901-527 was used as the IFN-α standard throughout.

Prevention of EBV-induced B-cell outgrowth by IFN-α. Cells were incubated at 37°C at the desired concentration in 1.0 ml of RPMI 1640 supplemented with 20% fetal calf serum, penicillin, streptomycin, HEPES buffer, and IFN-α in various concentrations (0, 20, 40, 100, 200, 500, 1,000, and 2,000 U/ml). Rarely, an insufficient number of cells were available and the highest IFN-α concentrations were deleted. After incubation in IFN-α for 18 to 20 h, cells were pelleted and resuspended in virus-containing medium at 37°C for 2 h with periodic gentle agitation. Whenever possible, cells incubated in virus-free medium were included in each assay. The cells were then pelleted, resuspended in medium containing the appropriate concentration of IFN-α, and distributed into wells (flat bottom) of a 96-well microtiter plate. In general, four replicate wells were prepared at each IFN-α concentration. Wells were fed by removing half of the medium and repleting with fresh medium containing the appropriate IFN-α concentration. Wells were fed after 7 days and twice weekly thereafter. Experiments were terminated when the presence or absence of transformation was apparent by microscopic examination, usually between 4 and 6 weeks. In most instances [^{3}H]thymidine uptake or EBNA positivity was used to confirm the presence of transformation.

DNA synthesis assay. One day after feeding, cells were pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and incubated at 37°C for 4 h. Cells were then harvested with a semiautomatic multiple sample harvester (Microbiological Associates Bioproducts, Walkersville, Md.) on glass fiber filters. Radioactivity was assayed by liquid scintillation counting, using Omnifluor scintillation fluid (New England Nuclear Corp.).

EBNA detection. The presence of EBNA in mononuclear cells was detected by anticomplementary immunofluorescence (17).

RESULTS

Standardization of assay conditions. Attempts were made to identify and control for important variables in the assay system utilized. The effects of varying (i) the concentration of CBMCs, (ii) the IFN-α treatment schedule used, or (iii) the viral inoculum were studied. Prevention of outgrowth was dependent on CBMC concentration (Fig. 1). In subsequent experiments, a mononuclear cell concentration of

between 1.6×10^6 and 2.2×10^6/ml was used. The timing and duration of IFN-α treatment were also found to influence results in this assay. Incubation with IFN-α for 18 to 20 h before infection with EBV, followed by feeding throughout the experiment with media containing the appropriate IFN-α concentration, was the most effective schedule for preventing outgrowth (mean of 100 U of IFN-α per ml required), although feeding with media containing IFN-α for just the first week was only slightly less successful (mean of 200 U of IFN-α per ml required). Removal of IFN-α at the time of infection with B95-8 was substantially less effective (mean of 1,750 U of IFN-α per ml required). The first regimen was used in all subsequent experiments. There was a positive correlation between the B95-8 inoculum used and the IFN-α concentration required to prevent outgrowth. Subsequently, a viral titer of $10^{2.0}$ to $10^{3.0}$ 50% transforming doses (TD_{50})/ml was used unless otherwise indicated. Figure 2 presents data from experiments comparing the IFN-α sensitivity of EBV-induced B-cell outgrowth, using CBMCs, T-cell-depleted CBMCs, and adult T-cell-depleted mononuclear cells. The mean IFN-α concentrations required to prevent outgrowth in all three cell preparations were similar.

IFN-α sensitivity of B-cells transformed by clinical EBV isolates. Throat wash or salivary specimens or both from 21 renal allograft recipients and 7 patients with infectious mononucleosis were tested for the presence of EBV. A total of 76% of the renal allograft recipients had EBV detected in their throat wash or saliva specimens; all patients with infectious mononucleosis had virus present. The highest titer in a renal allograft recipient was $10^{3.4}$ TD_{50}/ml; the highest titer in a patient with infectious mononucleosis was $10^{3.8}$ TD_{50}/ml.

Figure 3 compares the concentration of IFN-α required to prevent outgrowth of CBMCs after infection with B95-8 virus with that required to prevent outgrowth after infection with clinical EBV isolates. Clinical specimens with transforming titers of $<10^{1.0}$ TD_{50}/ml were not used since exposing CBMCs to these specimens in the absence of IFN-α did not consistently result in B-cell outgrowth in all wells. Throat wash specimens were run simultaneously with a titer-matched B95-8 specimen, using CBMCs from a single cord blood specimen. Isolates from patients with infectious mononucleosis and renal transplant recipients required similar concentrations of IFN-α to prevent outgrowth. The clinical isolates required slightly higher concentrations of IFN-α to prevent outgrowth than did the B95-8 virus, but the

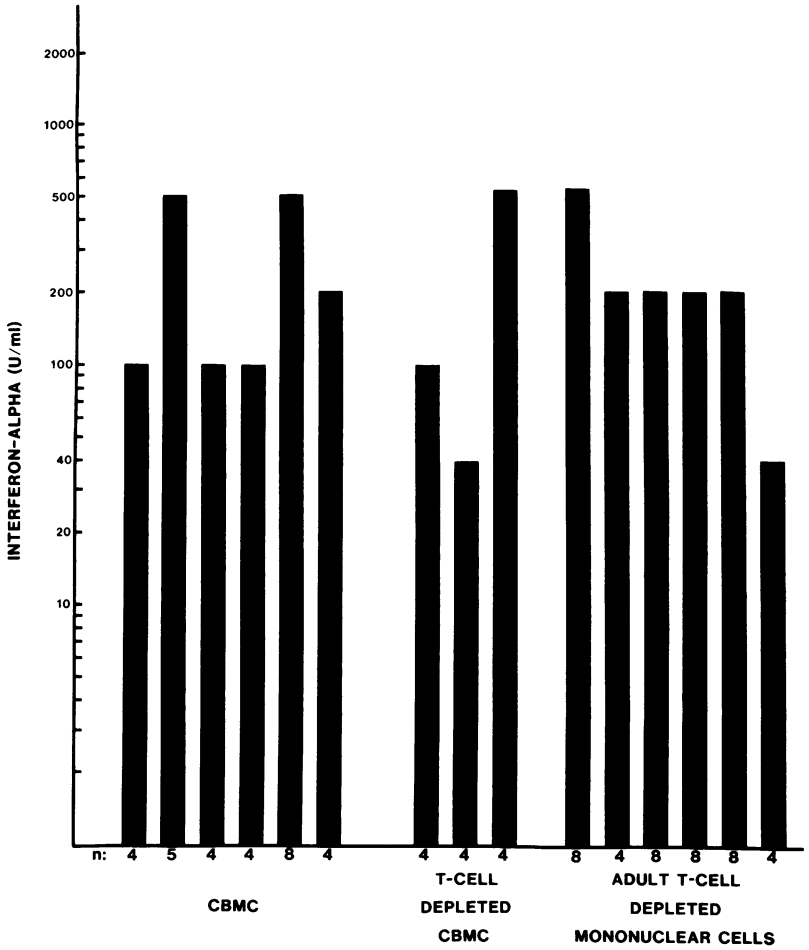

FIG. 2. Effect of IFN-α on B-cell outgrowth due to B95-8 infection of CBMCs, T-cell-depleted CBMCs, and adult T-cell-depleted mononuclear cells. Each bar graph represents mononuclear cells derived from a different individual and terminates at the first IFN-α concentration which prevented outgrowth in all replicate wells (n = number of replicate wells). Mean IFN-α concentrations required to prevent outgrowth were: CBMCs, 250 ± 16 U/ml; T-cell-depleted CMBCs, 213 ± 15 U/ml; adult T-cell-depleted mononuclear cells, 223 ± 15 U/ml.

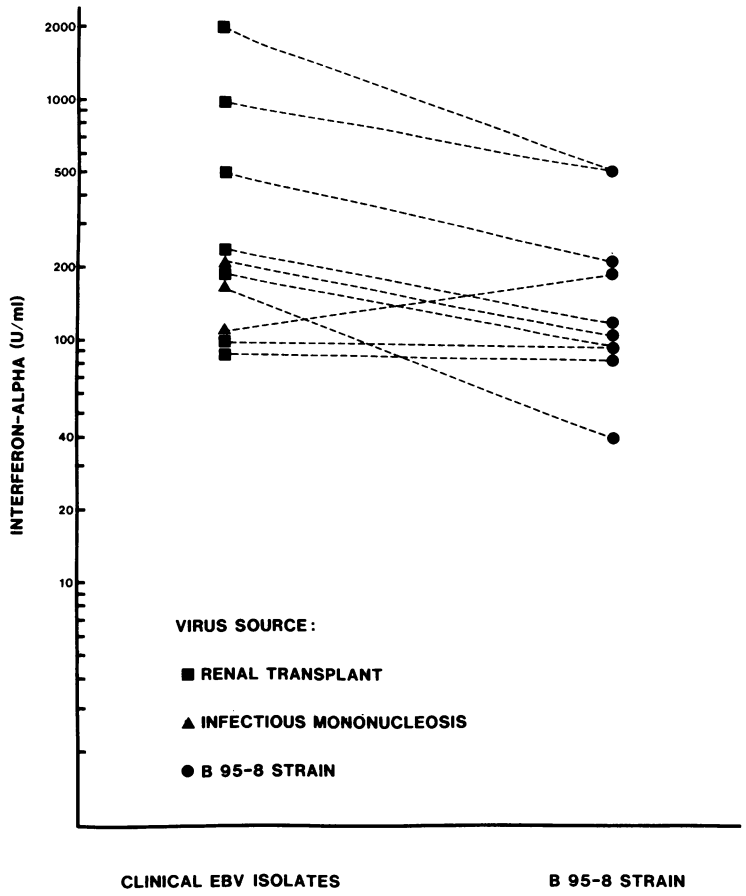

FIG. 3. IFN-α concentration required to prevent B-cell outgrowth after infection with clinical EBV isolates. Each clinical specimen was evaluated simultaneously with a titer-matched B95-8 specimen, and four replicate wells were set up at each IFN-α concentration. The symbol appears at the first IFN-α concentration which inhibited outgrowth in all four replicate wells. The concentrations required to prevent outgrowth induced by clinical EBV isolates are not significantly different from those required to prevent outgrowth due to B95-8 virus ($P > 0.05$ by paired t-test.

differences were not statistically significant ($P > 0.05$ by paired t-test). In general, there was a positive correlation between viral inoculum and the concentration of IFN-α required to prevent outgrowth. Titers used ranged from $10^{1.4}$ to $10^{2.8}$ TD_{50}/ml. In one instance, throat wash specimens from a renal allograft recipient were available before and after a course of prophylactic IFN-α administered as part of a separate study. The concentration of IFN-α required to prevent outgrowth was similar for both specimens (200 U/ml before IFN-α and 100 U/ml after IFN-α administration).

One mechanism by which IFN-α could prevent EBV-induced B-cell outgrowth would be via an antiproliferative effect. We incubated two recently transformed EBNA-positive cell lines in concentrations of IFN-α ranging up to 2,000 U/ml. Cells were fed biweekly with media containing IFN-α. After 3 weeks cells were counted and [^{3}H]thymidine uptake was determined. There was no effect on either parameter at any of the concentrations used.

DISCUSSION

We have defined three variables which influence the concentration of IFN-α required to prevent EBV-induced B-cell outgrowth: cell concentration, IFN-α treatment regimen, and viral inoculum. The influence of cell concentration is perhaps due to non-B-cells functioning as a feeder layer to promote survival of small numbers of EBV-transformed B-cells (5). It may also relate to the presence of a larger number of transformed B-cells when larger total cell numbers are used, but this seems less likely since an equal concentration of IFN-α was required to prevent outgrowth in the T-cell-depleted CBMC fraction and the unfractionated CBMCs. The treatment regimen used also had a significant effect on the concentration of IFN-α required to prevent outgrowth. IFN-α treatment throughout the entire experiment was the most effective regimen. IFN-α treatment for just the first week was only slightly less effective, suggesting that the primary impact of IFN-α treatment in this system occurs during the initial week after infection. Thorley-Lawson has reported that adult T-cells act, at least in part, via IFN to inhibit EBV-induced B-cell transformation and that this event occurs within the initial 2 to 3 days postinfection (20). The diminished efficacy which resulted when IFN-α was removed at the time of infection with EBV demonstrates that merely having B-cells in an antiviral state at the time of infection was much less effective in preventing outgrowth. Finally, the EBV inoculum clearly affected results. Other investigators have also noted an inoculum effect with IFN-α, in both non-EBV (7, 19) and EBV (10) systems. The report by Menezes et al. (11) stating that IFN-α has no effect on B-cell transformation in CBMCs may be due to their use of a higher transforming titer than we used.

We investigated the sensitivity of outgrowth of cord blood

B-cells to IFN-α after EBV infection since it has been reported that IFN-α is incapable of diminishing [^{3}H]thymidine uptake in EBV-infected cord blood B-cells (21). Our data show that IFN-α is equally successful in preventing outgrowth in CBMCs, T-cell-depleted CBMCs, and T-cell-depleted adult mononuclear cells. This finding is consistent with reports from other laboratories (2, 10) and indicates that IFN-α may be equally active against EBV in neonates and adults.

The throat wash and saliva specimens contained EBV titers similar to those reported by other investigators (9, 14). Saliva has been reported to yield somewhat higher titers than throat washings, but our patients had great difficulty producing sufficient volumes of saliva, and throat washings were used predominantly. Since it has been reported that the IFN-α sensitivity of clinical cytomegalovirus isolates was two- to fourfold greater than that of the Davis strain (15), we studied the IFN-α sensitivity of outgrowth due to infection with our clinical isolates compared with outgrowth due to B95-8 stock virus. The clinical isolates required higher IFN-α concentrations to prevent outgrowth, but this trend was not statistically significant. In a single instance, EBV-containing throat wash specimens of adequate titer were available both before and after a course of IFN-α. The concentration of IFN-α required to inhibit outgrowth did not increase after the course of IFN-α, suggesting that IFN-α resistance did not play a role in perpetuating viral excretion in this patient.

In an effort to determine the mechanism by which IFN-α prevents EBV-induced B-cell proliferation, the effect of interferon on proliferation of recently transformed B-cell lines was determined. The lack of effect on the proliferation of EBNA-positive cell lines after 3 weeks of incubation in IFN-α at concentrations as high as 2,000 U/ml suggests that IFN-α may be acting through prevention of transformation or through another mechanism at an early stage of infection rather than simply exerting an effect on proliferation.

We have used a microtiter system for determining the concentration of IFN-α required to prevent B-cell outgrowth after EBV infection. Results were similar whether CBMCs, T-cell-depleted CBMCs, or T-cell-depleted adult mononuclear cells were used. Although clinical isolates required somewhat higher IFN-α concentrations to suppress outgrowth, the concentrations were within the limits of IFN-α serum levels achieved after administration of IFN-α in humans (3). It is also possible that indirect effects of IFN-α, such as its immunomodulatory activity (1, 13), will augment the direct effect examined in these studies and lead to clinically significant activity against EBV at lower concentrations of IFN-α.

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